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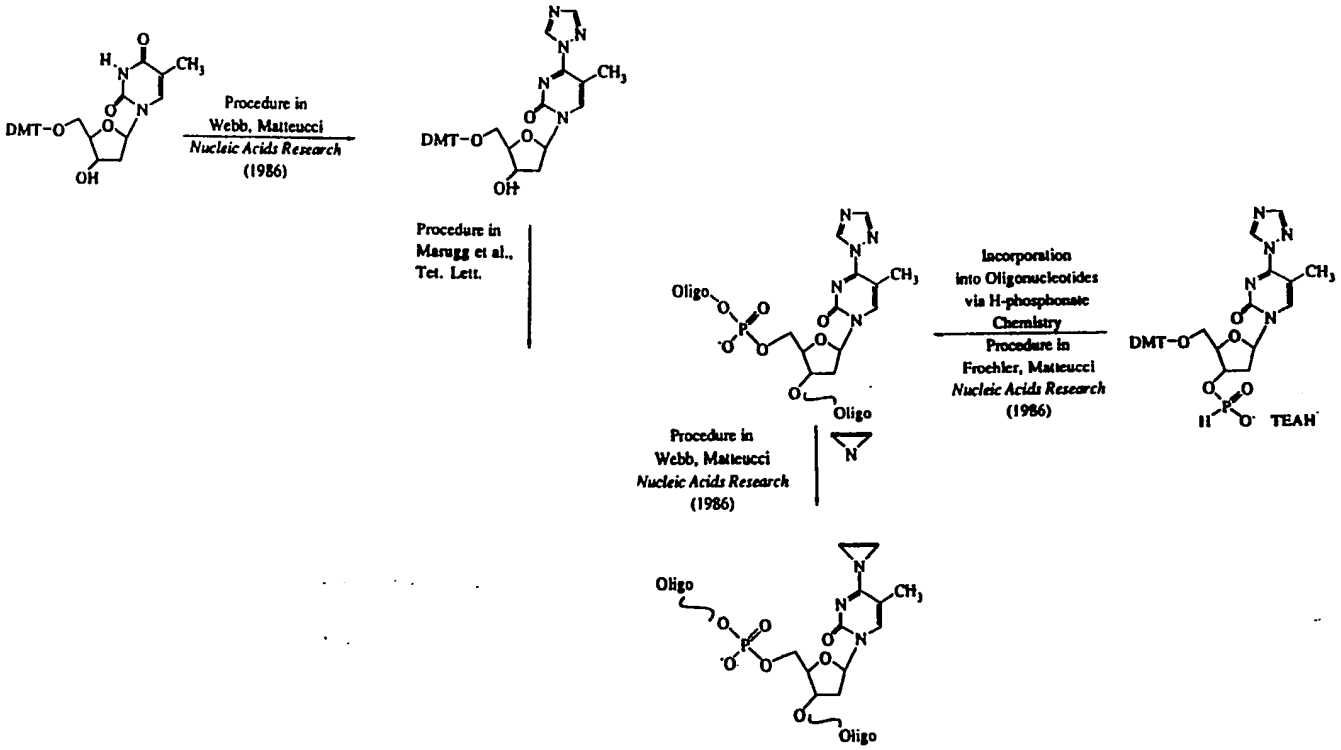

(57) Abstract

Novel triplexes, as well as methods to inhibit transcription by oligomeric binding to transcribed portions of DNA duplex transcription systems, are described. Several binding motifs can be used to effect triple helix formation, including the CT motif, the GT motif, as well as inverted polarity motifs.

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INHIBITION OF TRANSCRIPTION BY FORMATION OF TRIPLE HELIXES

Technical Field

The instant invention is directed to modes of inhibiting transcription using oligomers designed to form triple helices with duplex DNA. More specifically, the present invention concerns the interruption of transcription by triple helix formation between an oligomer and a transcribed region of duplex DNA.

Background of the Invention

DNA transcription is the process whereby RNA is synthesized from DNA using the enzyme RNA polymerase. RNA polymerase initiates the transcription process after binding to promoter DNA. The enzyme unwinds approximately one turn of the DNA helix to expose a stretch of single-stranded DNA that will act as a template for complementary base-pairing with incoming ribonucleotides. Two of the incoming ribonucleoside triphosphate monomers are then joined to begin an RNA chain. RNA polymerase travels along the DNA template strand, extending the growing chain in the 5'-to-3' direction, one nucleotide at a time. The enzyme continues to add nucleotides until a termination signal is reached, at which time the polymerase releases both the DNA template and the newly made RNA chain.
The instant invention provides a method for inhibiting this transcription proc ss by the formation of triple-helical complexes with transcribed regions of duplex DNA.

The rules which govern the association of single-stranded oligonucleotides with DNA duplexes to form triple-helical complexes have been recently described. At present, there are two recognized motifs for effecting triple helix formation. The older of these, commonly referred to as the "CT" motif, provides for a single-stranded oligomer containing, in its essential recognition portions, pyrimidine-based sequences. Pyrimidine oligomers bind purine-rich regions in the major groove, parallel to the purine strand of Watson-Crick double-helical DNA. Specificity is due to Hoogsteen hydrogen bonding where thymine (T) recognizes adenine-thymine (AT) base pairs and protonated cytosine (C) recognizes guanine-cytosine (GC) base pairs, resulting in T-A-T and C-G-C^+ based triplets, respectively, across the three associated chains of the resulting triple helix. This system is effective when there are long stretches in the duplex wherein one of the two strands contains only purine base residues. In summary, for formation of a triple helix which employs the CT mode, the oligomer will be designed to provide base "complementarity" to a purine-rich region of a single strand of the duplex in a parallel orientation to that strand. This mode of formation of triple helices has been summarized in the literature. See, e.g., Moser, H.E., and Dervan, P.B., Science (1987) 238:645-650.

An alternate motif, called the "GT" motif, was recognized more recently. In an article by Cooney, M., et al., Science (1988) 241:456-459 (see also European Patent Application No. 89313391.8), a purin -rich oligomer, containing a multiplicity of G residues, was reported to form a triplex with a DNA duplex which was rich in GC pairs and wherein the majority of the purine residues were located on a single strand of the targeted duplex upstream from the transcription start site. This

mode of association results in G-G-C triplets across the three strands in the triplex and A-A-T triplets were suggested, although not demonstrated. In this mode, again, the purine residues need to be concentrated on one strand of the duplex; however, the orientation of the oligomer with respect to the target duplex is reversed from that in the CT mode described above--i.e., the oligomer will be oriented in an antiparallel direction to the purine-rich strand of the duplex. The availability of the foregoing two motifs for triple helix formation offers expanded possibilities for the design of oligomers which are capable of triplex formation. It should be said, initially, that in all instances, a concentration of purine residues along a portion of a single strand of the targeted duplex is required. Nevertheless, it is advantageous to have available a repertoire of strategies for targeting duplexes which permits advantage to be taken of additional factors which may influence the stability of the resulting complex or the suitability of the administered oligomer.

Recently, additional strategies for triple helix formation have been proposed. These strategies are discussed at length in commonly owned, copending U.S. Application Serial Nos. 559,958 and 502,272, filed 30 July 1990 and 29 March 1990, respectively, and in commonly owned, copending PCT Application No. (Attorney Docket No. 4610-0007.40), filed 23 October 1990. Two of these strategies employ oligonucleotides which have inverted polarities for at least two regions of the oligonucleotide. By "inverted polarity" is meant that the oligonucleotide contains tandem sequences which have opposite polarity, i.e., one having polarity 5'→3' followed by another with polarity 3'→5', or vice versa. This implies that these sequences are joined by linkages which can be thought of as effectively a 3'-3'

internucleotide junction, (however the linkage is accomplished), or effectively a 5'-5' internucleotide junction. Such oligomers have been suggested as byproducts of reactions to obtain cyclic oligonucleotides by Capobianco, M.L., et al., Nucleic Acids Res (1990) 18:2661-2669. Compositions of "parallel-stranded DNA" designed to form hairpins secured with AT linkages using either a 3'-3' inversion or a 5'-5' inversion have been synthesized by van de Sande, J.H., et al., Science (1988) 241:551-557. In addition, triple helix formation using an oligomer which contains an effective 3'-3' linkage has been described by Horne, D.A. and Dervan, P.B., J Am Chem Soc (1990) 112:2435-2437.

Described below are methods of interrupting transcription by triple helix formation in promoter regions of transcribed DNA. Maher III, L.J., et al., Science (1989) 245:725-730, describe oligonucleotides that bind duplex DNA to form triple helices, which, in turn, inhibit transcription by blocking recognition of double helical DNA by prokaryotic modifying enzymes and a eukaryotic transcription factor. However, the inhibiting oligonucleotides bind at regulatory and not transcribed regions of the DNA duplex.

Similarly, some drugs, such as actinomycin D, are known to inhibit RNA synthesis. However, many of these substances act by intercalation, i.e., by insertion of the molecule between base pairs rather than by the formation of a triple helix via hydrogen bonds. Furthermore, these drugs often have harmful side effects due to nonspecific intercalation into DNA.

It is known that as RNA polymerase transcribes DNA, the molecule unwinds. The hydrogen bonding between base pairs does not interfere with transcription. Therefore, it is surprising that the formation of a triplex via hydrogen bonding can inhibit transcription.

Furthermore, although a few site-specific DNA binding proteins have been shown to prevent elongation of RNA by RNA polymerase (see e.g. Pavco, P.A. and Steege, D.A., J. Biol. Chem. (1990) 265:9960-9969; Sellitti, M.A., et al., Proc. Natl. Acad. Sci. (USA) (1987) 84:3199-3203), this enzyme generally is able to proceed with transcription despite the presence of bound proteins. Thus, it is quite unexpected that transcription can be inhibited by triplex formation between an oligomer and such transcribed regions. The ability to design oligomers that form triple helices with transcribed regions of DNA has major implications for the treatment of genetic, neoplastic, and viral diseases. The use of transcribed regions as targets for such oligomers makes available a huge array of sequences for use in such therapy.

Disclosure of the Invention

The instant invention is based on the discovery that certain oligomers are able to interfere with transcription by binding transcribed regions, including introns, exons, or both, of duplex DNA and forming triple helices. The design of oligonucleotide sequences to target transcribed regions of double-stranded duplex DNA can employ any of the above-described binding motifs, including the CT motif, the GT motif, and the use of oligonucleotides with inverted polarity, depending on the nature of the target duplex and other conditions characterizing the particular circumstance of administration of the oligomer.

Four basic situations can be envisioned. First, in a very simple case, understood in the art, the binding motif will be consistent along a standard oligomer of a single polarity. In this case, only a single strand of the duplex can be targeted, and while

more than one purine-rich region along this strand can be engaged in triplex formation with oligomers of this type, such plurality of regions may be interrupted by sections of pyrimidine enrichment where no association occurs.

In a second situation, it may be desirable to target purine-rich transcribed regions along a single strand of a duplex but to provide bonding using different motifs. For example, the GT motif may offer some advantages when guanine-rich regions are encountered because of the pH dependence of binding of the cytosine residues in the oligomer. Thus, homopurine regions rich in guanidine may advantageously be coupled using the GT motif. Alternatively, guanine-rich targets may also be bound utilizing appropriate modified nucleotides such as N6-methyl-8-hydroxy-2'-deoxyadenosine, described in commonly owned, copending U.S. Patent Application Serial No. (Attorney Docket No. 4610-0018), filed 23 November 1990, which efficiently recognize guanine residues under physiological conditions. On the other hand, it may be desirable to access adenine-rich regions using the CT motif. A change in motif of binding along a single strand of the duplex must be coupled with an inversion of polarity in the oligomer to correspond in the change in motif. Such oligomers are described in detail in commonly owned, copending PCT Application No. (Attorney Docket No. 4610-0007.40), filed 23 October 1990.

In the third and fourth situations, it may be desired to cross over and switch back and forth between purine-rich regions on opposite strands of the duplex. This can be done, for a single crossover, by either inverting the polarity of the targeting oligonucleotide using a constant motif or by maintaining the polarity constant and changing the binding motif.

Thus, in some instances, the ability of oligonucleotide sequences to hybridize to double-stranded

duplex DNA is enhanced by providing oligonucleotides with inverted polarity either so that the binding oligonucleotide can skip from one complementary strand in the duplex to the other as its polarity shifts, or so that advantage can be taken of the alternate motif. In its simplest embodiment, there is a single inversion of polarity in the binding oligonucleotide; of course, inversions can be inserted in a number depending on the DNA duplex target sequence.

Thus, in one aspect, the instant invention relates to a method to inhibit transcription of duplex DNA. The method comprises contacting duplex DNA having a control region and a transcribed region with an amount of oligomer effective to bind to the transcribed region, thereby forming a triple helix.

In another embodiment the invention is directed to a method of inhibiting RNA polymerase by a major groove binding agent. The method comprises binding the major groove binding agent to transcribed regions of DNA in a sequence-specific manner.

In yet another embodiment, the present invention is directed to a DNA triplex comprising a) duplex DNA having a control region and a transcribed region; and b) a single-stranded DNA oligomer coupled to a portion of the transcribed region.

These and other embodiments of the present invention will readily occur to those of ordinary skill in the art in view of the disclosure herein.

Brief Description of the Figures

Figure 1 depicts the synthesis scheme for the base analog aziridinylcytosine.

Detailed Description

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, and recombinant DNA, which are within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Maniatis, et al., Molecular Cloning: A Laboratory Manual (1982); DNA Cloning (1985) Vols. I and II, D.N. Glover (ed.); Nucleic Acid Hybridization (1984), B.D. Hames, et al. (eds.); Perbal, B., A Practical Guide to Molecular Cloning (1984); Methods in Enzymology (the series), Academic Press, Inc.; Vectors: A Survey of Molecular Cloning Vectors and Their Uses (1987), R.L. Rodriguez, et al., (eds.), Butterworths; and Miller, J.H., et al., Experiments in Molecular Genetics (1972) Cold Spring Harbor Laboratory.

All patents, patent applications, and publications mentioned herein, whether supra or infra, are hereby incorporated by reference.

As used herein "oligonucleotide" is generic to polydeoxyribonucleotides (containing 2'-deoxy-D-ribose or modified forms thereof), i.e., DNA, to polyribonucleotides (containing D-ribose or modified forms thereof), i.e., RNA, and to any other type of polynucleotide which is an N-glycoside or C-glycoside of a purine or pyrimidine base, or modified purine or pyrimidine base.

By "major groove binding agent" is meant an oligonucleotide which binds DNA in a sequence-specific manner in the major groove of the DNA duplex. By "sequence-specific manner" is meant that the oligonucleotide used for binding recoginizes defin d sequences of transcribed regions of DNA, as opposed to regulatory regions. When bound, the oligonucleotide serves to inhibit RNA polymerase (see, Sawadogo, M. and

Roeder, R.G., Proc. Natl. Acad. Sci (USA) (1985) 82:4394-4398, and in turn, interrupts transcription.

The term "nucleoside" or "nucleotide" will similarly be generic to ribonucleosides or ribonucleotides, deoxyribonucleosides or deoxyribonucleotides, or to any other nucleoside which is an N-glycoside or C-glycoside of a purine or pyrimidine base, or modified purine or pyrimidine base. Thus, the stereochemistry of the sugar carbons may be other than that of D-ribose in certain limited residues, as further described below.

"Nucleoside" and "nucleotide" include those moieties which contain not only the known purine and pyrimidine bases, but also heterocyclic bases which have been modified. Such modifications include alkylated purines or pyrimidines, acylated purines or pyrimidines, or other heterocycles. Such "analogous purines" and "analogous pyrimidines" are those generally known in the art, many of which are used as chemotherapeutic agents. An exemplary but not exhaustive list includes 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyl uracil, dihydrouracil, inosine, N6-isopentenyl-adenine, 1-methyladenine, 1-methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-methyladenine, 7-methylguanine, 5-methylaminomethyl uracil, 5-methoxy aminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, wybutoxosine, pseudouracil, queosine, 2-thio cytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, and

2,6-diaminopurine. "Nucleosides" or "nucleotides" also include those which contain modifications in the sugar moiety, for example, wherein one or more of the hydroxyl groups are replaced with halogen, aliphatic groups, or functionalized as ethers, amines, and the like. Examples of modified nucleosides or nucleotides include, but are not limited to:

2-aminoadenosine	2'-deoxy-2-aminoadenosine
5-bromouridine	2'-deoxy-5-bromouridine
5-chlorouridine	2'-deoxy-5-chlorouridine
5-fluorouridine	2'-deoxy-5-fluorouridine
5-iodouridine	2'-deoxy-5-iodouridine
5-methyluridine	(2'-deoxy-5-methyluridine is the same as thymidine)
inosine	2'-deoxy-inosine
xanthosine	2-deoxy-xanthosine

Furthermore, as the α anomer binds to duplexes in a manner similar to that for the ß anomers, one or more nucleotides may contain this linkage or a domain thereof. (Praseuth, D., et al., Proc Natl Acad Sci (USA) (1988) 85:1349-1353).

Other modified nucleotide residues which contain a substituent which provides hydrogen bonding donor and acceptor patterns compatible with Hoogsteen binding to duplex DNA can also be used. Exemplary are the modified residues described in commonly owned, copending U.S. Patent Application Serial No. (Attorney Docket No. 4610-0018), filed 23 November 1990, such as N6-methyl-8-hydroxy-2'-deoxyadenosine. This base analog is particularly useful since it binds in both the GT and CT motifs. Pseudonucleotides such as anthraquinone derivatized oligonucleotides (described in commonly owned, copending U.S. Patent Application Serial Nos.

594,147 and 482,941, filed 9 October 1990 and 20 February 1990, respectively) will also find use in the instant method as will DNA alkylating base analogs, such as aziridinyl nucleotides, described in commonly owned, copending U.S. Patent Application Serial No. 529,346, filed 25 May 1990.

The Binding Oligonucleotides

The oligonucleotides for use in binding transcribed DNA regions may be of any length, but lengths of greater than or equal to about 10 nucleotides, preferably about 10 to about 25 nucleotides are preferred. However, longer oligonucleotides may also be made, particularly those of greater than 50 nucleotides or greater than 100 nucleotides.

Oligonucleotides may contain conventional internucleotide phosphodiester linkages or may contain modified forms such as phosphoramidate linkages. These alternative linking groups include, but are not limited to embodiments wherein a moiety of the formula P(O)S, $P(O)NR_2$, P(O)R, P(O)OR', CO, or $CONR_2$, wherein R is H (or a salt) or alkyl (1-12C) and R' is alkyl (1-6C) is joined to adjacent nucleotides through -O- or -S-. Formacetal/ketal type linkages are further disclosed in commonly owned, copending U.S. Patent Application Serial No. 557,957, filed 30 July 1990. In addition, specifically included are the 2'-derivatized forms of the nucleotide residues disclosed in commonly owned, copending U.S. Patent Application Serial No. 425,857, filed 24 October 1989. Other useful modified linkers include linkages composed of two to four atoms wherein at least one of the atoms making up the linkage is nitrogen, oxygen or sulfur, with the remainder being carbon. Exemplary are linkers having the formula -CH2-CH2-NH-, and -O-CH2-CH2-NH-. Such modified linkages are described in

detail in commonly owned, copending U.S. Patent Application Serial No. 585,780, filed 20 September 1990. Not all of the above described linkages in the same oligomer need to be identical.

Oligomers used in binding transcribed sequences, including those involving inversions of polarity, can be "derivatives" of oligonucleotides. "Derivatives" of the oligomers include those conventionally recognized in the art. For instance, the oligonucleotides may be covalently linked to various moieties such as intercalators, substances which interact specifically with the minor groove of the DNA double helix and other arbitrarily chosen conjugates, such as labels (radioactive, fluorescent, enzyme, etc.). These additional moieties may be derivatized through any convenient linkage. For example, intercalators, such as acridine can be linked through any available -OH or -SH, e.g., at the terminal 5' position of RNA or DNA, the 2' positions of RNA, or an OH, NH_2, COOH or SH engineered into the 5 position of pyrimidines, e.g., instead of the 5 methyl of cytosine, a derivatized form which contains, for example, $-CH_2CH_2NH_2$, $-CH_2CH_2CH_2OH$ or $-CH_2CH_2CH_2SH$ in the 5 position. A wide variety of substituents can be attached, including those bound through conventional linkages. The indicated -OH moieties in the oligomers may be replaced by phosphonate groups, protected by standard protecting groups, or activated to prepare additional linkages to other nucleotides, or may be bound to the conjugated substituent. The 5' terminal OH may be phosphorylated; the 2'-OH or OH substituents at the 3' terminus may also be phosphorylated. The hydroxyls may also be derivatized to standard protecting groups.

Oligonucleotides or the segments thereof of 5'→3' or 3'→5' polarity, i.e. those useful in the CT and GT motifs, are conventionally synthesized. Methods for

such synthesis are found, for example, in Froehler, B., et al., Nucleic Acids Research (1986) 14:5399-5467; Nucleic Acids Research (1988) 16:4831-4839; Nucleosides and Nucleotides (1987) 6:287-291; Froehler, B., Tet Lett (1986) 27:5575-5578.

In general, there are two commonly used solid phase-based approaches to the synthesis of oligonucleotides containing conventional 3'→5' or 5'→3' linkages, one involving intermediate phosphoramidites and the other involving intermediate phosphonate linkages. In the phosphoramidite based synthesis, a suitably protected nucleotide having a cyanoethylphosphoramidite at the position to be coupled is reacted with the free hydroxyl of a growing nucleotide chain derivatized to a solid support. The reaction yields a cyanoethyl-phosphite, which linkage must be oxidized to the cyanoethylphosphate at each intermediate step, since the reduced form is unstable to acid. The H-phosphonate-based synthesis is conducted by the reaction of a suitably protected nucleoside containing an H-phosphonate moiety at a position to be coupled with a solid phase-derivatized nucleotide chain having a free hydroxyl group, in the presence of a suitable activator to obtain an H-phosphonate diester linkage, which is stable to acid. Thus, the oxidation to the phosphate or thiophos-phate can be conducted at any point during the synthesis of the oligonucleotide or after synthesis of the oligonucleotide is complete. The H-phosphonates can also be converted to phosphoramidate derivatives by reaction with a primary or secondary amine in the presence of carbon tetrachloride. To indicate the two approaches generically, the incoming nucleoside is regarded as having an "activated phosphite/phosphate" group.

Variations in the type of internucleotide linkage are achieved by, for example, using the methyl

phosphonate precursors rather than the H-phosphonates per se, using thiol derivatives of the nucleoside moieties and generally by methods known in the art. Nonphosphorous based linkages may also be used, such as the modified linkers described above.

Thus, to obtain an oligonucleotide segment which has a 3'→5' polarity, a nucleotide protected at the 5' position and containing an activated phosphite/phosphate group at the 3' position is reacted with the hydroxyl at the 5' position of a nucleoside coupled to a solid support through its 3'-hydroxyl. The resulting condensed oligomer is deprotected and the reaction repeated with an additional 5'-protected, 3-'phosphite/phosphate activated nucleotide. Conversely, to obtain an oligomeric segment of 5'→3' polarity, a nucleotide protected in the 3' position and containing an activated phosphite/phosphate in the 5' position is reacted with a nucleotide oligomer or nucleoside attached to a solid support through the 5' position, leaving the 3'-hydroxyl available to react. Similarly, after condensation of the incoming nucleotide, the 3' group is deprotected and reacted with an additional 3'-protected, 5'-activated nucleotide. The sequence is continued until the desired number of nucleotides have been added.

In addition to employing these very convenient and now most commonly used, solid phase synthesis techniques, oligonucleotides may also be synthesized using solution phase methods such as triester synthesis. These methods are workable, but in general, less efficient for oligonucleotides of any substantial length.

The oligonucleotides of the invention which are designed to target transcribed duplexes for triplex formation may have, as stated above, either uniform or mixed motifs and may or may not contain regions of inverted polarity. With respect to variation in motif,

portions of the oligomers may contain regions designed to effect CT-type binding; additional regions in the same oligomer are designed to effect GT-type binding. The regions designed to effect CT-type binding will be enriched in pyrimidine residues, as in this mode, the T-residues can be considered to target A-residues and C-residues can be considered to target G-residues in the duplex and the targeted purine regions are read in an antiparallel orientation with respect to the oligonucleotide. A region of the oligonucleotide of the invention which effects CT binding will be pyrimidine rich and will contain a sequence designed to read a purine-enriched sequence on a strand of a target duplex in a parallel orientation.

Where all of the oligonucleotide is designed to effect the GT motif, of course, the binding sequences will be enriched in G-residues. Thus, for mixed motif oligonucleotides, regions of pyrimidine enrichment will alternate with purine enrichment regions. Where the alteration in motif is combined with inverted polarity, in general, the transition from pyrimidine enrichment to, for example, purine enrichment will generally coincide with the region of inversion of polarity.

Whatever the design of the oligonucleotide, the strand of the duplex containing a purine-enriched region is formally targeted in order to rationalize the rules. It is understood that this "targeting" of the purine-rich region is a matter of formality and the rules could be rewritten in terms of the pyrimidine-enriched strand, if desired.

Inverted polarity oligonucleotides, in their most general form, contain at least one segment along their length of the formula:

3'----->5'--C--5'-----3' (1)

or

5'----->3'--C--3'-----5' (2)

where -C- symbolizes any method of coupling the nucleotide sequences of opposite polarity.

In these formulas, the symbol 3'----5' indicates a stretch of oligomer in which the linkages are consistently formed between the 5' hydroxyl of the ribosyl residue of the nucleotide to the left with the 3' hydroxyl of the ribosyl residue of the nucleotide to the right, thus leaving the 5' hydroxyl of the rightmost nucleotide ribosyl residue free for additional conjugation. Analogously, 5'----3' indicates a stretch of oligomer in the opposite orientation wherein the linkages are formed between the 3' hydroxyl of the ribosyl residue of the left nucleotide and the 5' hydroxyl of the ribosyl residue of the nucleotide on the right, thus leaving the 3' hydroxyl of the rightmost nucleotide ribosyl residue free for additional conjugation.

The linkage, symbolized by -C-, may be formed so as to link the 5' hydroxyls of the adjacent ribosyl residues in formula (1) or the 3' hydroxyls of the adjacent ribosyl residues in formula (2), or the "-C-" linkage may conjugate other portions of the adjacent nucleotides so as to link the inverted polarity strands. "-C-" may represent a linker moiety, or simply a covalent bond.

It should be noted that if the linkage between strands of inverted polarity involves a sugar residue,

either the 3' or 2' position can be involved in the linkage, and either of these positions may be in either R or S configuration. The choice of configuration will in part determine the geometry of the oligomer in the vicinity of the linkage. Thus, for example, if adjacent 3' positions are used to effect a covalent linkage, less severe deformation of the oligonucleotide chain will generally occur if both 3' hydroxyls involved in the linkage are in the conventional R configuration. If they are both in the S configuration, this will result in a "kink" in the chain.

In addition to the use of standard oligonucleotide synthesis techniques or other couplings to effect the 5'-5' or 3'-3' linkage between ribosyl moieties, alternative approaches to joining the two strands of inverted polarity may be employed. For example, the two appended bases of the opposing termini of the inverted polarity oligonucleotide sequences can be linked directly or through a linker, or the base of one can be linked to the sugar moiety of the other. Any suitable method of effecting the linkage may be employed. The characterizing aspect of the switchback oligonucleotides of the invention is that they comprise tandem regions of inverted polarity, so that a region of 3'→5' polarity is followed by one of 5'→3' polarity, or vice versa, or both.

Depending on the manner of coupling the segments with inverted polarity, this coupling may be effected by insertion of a dimeric nucleotide wherein the appropriate 3' positions of each member of the dimer or the 5' positions of ach member of the dimer are activated for inclusion of the dimer in the growing chain, or the conventional synthesis can be continued but using for the condensing nucleotide a nucleotide which is protected/activated in the inverse manner to that which would be

employed if the polarity of the chain were to remain the same. This additional nucleotide may also contain a linker moiety which may be included before or after condensation to extend the chain.

When a linker moiety is employed, a phosphite/ phosphate activated linker can be included directly in the continuing oligonucleotide synthesis, followed by coupling to the first nucleotide of the inverted sequence or the first such nucleotide can be supplied already derivatized to the phosphite/ phosphate activated linker. In general, the linker comprises a diol or diamine. Thus, in a typical synthesis protocol, one hydroxyl (or amino) of the diol (or diamine) is protected and the other is an activated phosphite/phosphate. This protected form can be coupled to the oligonucleotide chain attached to the solid support and then deprotected and reacted with the subsequent nucleotide residue.

Similar diol or diamine type (or disulfhydryl or hydroxyl/sulfhydryl type) linkers are also convenient when the linkage between inverted polarity segments is to be effected between adjacent bases or between a base and a sugar moiety, or these can be used to link adjacent sugars directly without the inclusion of the phosphodiester or analog thereof. In these instances, it is generally more convenient to synthesize the switchback nucleotide dimer independently, and then to insert the dimer, again using standard oligonucleotide synthesis techniques, into the oligonucleotide to be formed. Alternate linker functionalities can be convenient when adjacent base moieties are to be used, however, in general, convenient forms of linkers are those derived from dihydroxy, diamino (or disulfhydryl or hydroxyl/ sulfhydryl) compounds which can be suitably protected and activated so as to integrate them into the standard

oligonucleotide synthesis protocol or otherwise used to obtain inverted dimeric nucleotides.

The significant step in the integration of these linkers, however, is that the subsequent additions to the oligomer, after the linker is inserted, are activated and deprotected nucleotides having opposite polarity from that of the preceding portion of the sequence.

The length and type of internucleotide linkage at the inverted junction will depend in part on the charge concentration (e.g., polyphosphodiester groups may be too highly concentrated in charge) and on the distance required to span the major groove in the duplex in order to achieve the required triple helix binding. It is presently considered that spanning the two strands of the duplex through a 5'-5' switchback involves no null bases, while a 3'-3' switchback involves 1-4 null bases in the duplex target. (A "null" base refers to a base pair in the DNA duplex that does not hydrogen bond to the third strand moiety.) The length of the linker can be adjusted accordingly. The proper length and type of linkage may be determined by those of ordinary skill in the art using routine optimization procedures. Inverted polarity oligomers for use with the instant invention are described in detail in commonly owned, copending U.S. Patent Application Serial No. 559,958, filed 30 July 1990, and in commonly owned, copending PCT Application No. (Attorney Docket No. 4610-0007.40), filed 23 October 1990.

The synthesis of oligonucleotides having inverted polarity may be accomplished utilizing standard solid phase synthesis methods described above and known in the art. Oligonucleotide chain longation will pr ceed in conformance with a predetermined sequence in a series of condensations, each one of which results in the addition of another nucleotide. Prior to the addition of

a nucleoside having an activated phosphite/ phosphate, the protecting group on the solid support-bound nucleotide is removed. Typically, for example, removal of the commonly-employed dimethoxytrityl (DMT) group is done by treatment with 2.5% v/v dichloroacetic acid/ dichloromethane, although 1% w/v trichloroacetic acid/ dichloromethane or $ZnBr_2$-saturated nitromethane, are also useful. Other deprotection procedures suitable for other protecting groups will be apparent to those of ordinary skill in the art. The deprotected nucleoside or oligonucleotide bound to solid support is then reacted with the suitably protected nucleotide containing an activated phosphite/ phosphate. After each cycle the carrier bound nucleotide is preferably washed with anhydrous pyridine/ acetonitrile (1:1, v/v), again deprotected, and the condensation reaction is completed in as many cycles as are required to form the desired number of congruent polarity internucleotide bonds which will be converted to phosphoramidates, phosphorodithioates, phosphorothioates or phosphodiesters as desired.

In one method to provide the switchback, the incoming activated, protected nucleoside is provided in the opposite polarity to the support-bound oligomers. Thus, for example, where the support-bound oligomer is 3'→5', the deprotected 5' hydroxyl is reacted with a 3'-protected, 5'-activated monomer, and the synthesis continued with monomers activated at the 5' position and protected at the 3' position.

In another method to provide a linker in the switchback, a molecule having one end which is activated for condensation (such as a hydrogen phosphonate) to the support-bound oligonucleotide and another end which is a protected hydroxyl group (or protected thio group) is condensed onto the support-bound oligonucleotide. The linker group is condensed and deprotected using the same

conditions as those used to condense and deprotect the protected nucleoside hydrogen phosphonate. Subsequent extension of the oligonucleotide chain then uses oligonucleotide residues which are activated and protected in the opposite manner from those used to synthesize the previous portion of the chain.

It will be understood that while the above method has been described in connection with use of a solid state carrier, it is also possible to conduct the synthesis without the use of a solid state support. In such an event, in place of the support a 3'-hydroxy protecting group which is different from the 5' protecting group used in the course of the condensation, may be utilized so that the 5' protecting group may be selectively removed while the 3' protecting group remains intact.

Binding Properties

The oligonucleotides for use with the instant invention, including those with inverted polarity, are designed to effect triplex formation with target transcribed oligonucleotide duplexes, thereby inhibiting transcription of the same. In vitro conditions for the triplex formation are variable, but in order to be maximally effective in use for the treatment of disease or for analysis as described hereinbelow, it is generally preferred that the triplex formation be effected under simulated physiological salt, pH and temperature conditions. The manner of triplex formation will depend, of course, on the design of the oligonucleotide.

It will be understood that those oligomers which are designed to participate in CT-type motifs will contain, in those regions intended for CT binding, mostly pyrimidine-based nucleotides. Those regions of the oligonucleotides intended to participate in GT-type

binding will contain mainly purine-type nucleotides. For oligonucleotides which maintain the same binding motif but are intended to cross over between the strands of the target duplex, an inversion of polarity will be provided. Thus, the nucleotides will comprise, on one side of the 3'-3' (or 5'-5') inversion, bases which bind to one strand of the duplex according to the motif chosen, with the bases on the other side of the 3'-3' (or 5'-5') junction selected to be bases which will bind to the subsequent bases on the opposite strand of the duplex according to the same motif.

In this manner triple helix recognition may be extended by switching recognition from one strand of the duplex to the other and then back again, if desired. Also, certain nucleases may be blocked, since the oligonucleotides according to the present invention can present ends not recognizable by exonucleases. Thus, oligonucleotides having two 5'-ends, will be resistant to 3'-exonucleases.

The ability of the above described oligomers to inhibit transcription can be tested using any standard transcription inhibition assay. Such assays are known in the art and include the triplex inhibition of transcription assay described in the examples, as well as nuclear run-off assays (see e.g. Sawadogo, M. and Roeder, R.G., Proc. Natl. Acad. Sci. (USA) (1985) 82:4394-4398), and nuclease S1 assays (see e.g. Berk, A. and Sharp, P., Cell (1977) 12:721-732, among others. The ability to target transcribed regions to inhibit transcription, as opposed to targeting regulatory nucleotide regions, tremendously increases the sequences available for targeting and provides a more efficient method to modulate gene expression.

Utility and Administration

As the oligonucleotides for use with the instant invention are capable of significant duplex binding activity to form triplexes or other forms of stable association with transcribed regions, these complexes are useful in "antisense" therapy. "Antisense" therapy as used herein is a generic term which includes the use of specific binding oligonucleotides to inactivate undesirable DNA or RNA sequences in vitro or in vivo.

Most diseases and other conditions are characterized by the presence of undesired DNA or RNA, some of which may be in duplex form. These diseases and conditions can be treated using the principles of antisense therapy as is generally understood in the art. Antisense therapy includes targeting a specific DNA or RNA sequence through complementarity or through any other specific binding means, in the case of the present invention by sequence-specific orientation in the major groove of the DNA double helix of transcribed regions.

The oligomers for use in the instant invention may be administered singly, or combinations of oligomers may be administered for adjacent or distant targets or for combined effects of antisense mechanisms with the foregoing general mechanisms.

In therapeutic applications, the oligomers can be formulated for a variety of modes of administration, including systemic, topical or localized administration. Techniques and formulations generally may be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA, latest edition. The oligomer active ingredient is generally combined with a carrier such as a diluent or excipient which may include fillers, extenders, binders, wetting agents, disintegrants, surface-active agents, or lubricants, depending on the nature of the mode of administration and dosage forms.

Typical dosage forms include tablets, powders, liquid preparations including suspensions, emulsions and solutions, granules, capsules and suppositories, as well as liquid preparations for injections, including liposome preparations.

For systemic administration, injection is preferred, including intramuscular, intravenous, intraperitoneal, and subcutaneous. For injection, the oligomers for use with the invention are formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the oligomers may be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included.

Systemic administration can also be by transmucosal or transdermal means, or the compounds can be administered orally. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, bile salts and fusidic acid derivatives for transmucosal administration. In addition, detergents may be used to facilitate permeation. Transmucosal administration may be through use of nasal sprays, for example, or suppositories. For oral administration, the oligomers are formulated into conventional oral administration forms such as capsules, tablets, and tonics.

For topical administration, the oligomers for use in the invention are formulated into ointments, salves, gels, or creams, as is generally known in the art.

In addition to use in therapy, the method of the present invention may be used diagnostically to detect the presence or absence of the target DNA or RNA sequences to which oligomers specifically bind. Such

diagnostic tests are conducted by hybridization through triple helix formation which is then detected by conventional means. For example, oligomers may be labeled using radioactive, fluorescent, or chromogenic labels and the presence of label bound to solid support detected. Alternatively, the presence of a triple helix may be detected by antibodies which specifically recognize these forms. Means for conducting assays using such oligomers as probes are generally known.

Experimental

The following examples are provided to illustrate but not to limit the invention.

Oligonucleotides (ODNs) utilized in the following examples are listed in Table 1. ODN 1 and 2 bind in the GT motif and all others bind in the CT motif. Standard solid phase techniques were used to synthesize the ODNs unless otherwise indicated.

Table 1

	ODN Sequence	Target Sequence
ODN 1	5′ GGG-GTT-GGT-GGG-GTG-T 3′	3′ GGG-GAA-GGA-GGG-GAG-A 5′
ODN 2	5′ GGT-GGT-GGT-GGT-GTT 3′	none, control ODN
ODN 3	5′ TZT-ZZZ-ZTZ-ZTT-ZZZ-X 3′	5′ AGA-GGG-GAG-GAA-GGG 3′
ODN 4	5′ WTW-TWT-TWT-ZWT-WTT-WT	5′ GAG-AGA-AGA-GGA-GAA-GA 3′
ODN 5	5′ YTT-CCT-CTC-TTC-TCC-TCT-TX	5′ GAA-GGA-GAG-AAG-AGG-AGA-A
ODN 6	5′ GGT-GTG-TTT-GTG-GGT-GTG-TTG	none, control ODN
ODN 7	5′ TWT-WTW-TWT-TTT-TT 3′	none, control ODN

ODN = oligomer.
Z = N6-methyl-8-hydroxy-2′-deoxyadenosine (described in Example 1a).
Y = aziridinylcytosine (described in Example 1b).
X = anthraquinone/pesudonucleotide (described in Example 1c).
W = 5-methylcytosine.

In the examples, the ability of the ODNs to block transcription was assessed using the following method.

A. Stimulated Jurkat Nuclear Extract Prep:

The nuclear protein extract for use in the triplex inhibition of transcription assay was prepared as described by Gorski, K., et al., Cell (1986) 47:767-776, with the following changes:

1) The cells were grown in RPMI1640 with glutamine, 10% fetal bovine serum, and the cells were stimulated with only 20 ng/ml PMA for three hours.

2) The protease inhibitors used were 4.5 μM leupeptin, 0.3 μM aprotinin, and 0.23 mM PMSF.

3) The homogenization volume and the volume of the sucrose gradient to pellet the nuclei was reduced by 1/2.

4) The nuclear proteins were precipitated at 20,000 RPM for twenty minutes.

B. Triplex Inhibition of Transcription Assay:

A 280 bp version of the Adenovirus major late promoter driven G-less cassette (Sawadogo, et al., Proc. Natl. Acad. Sci. (USA) (1985) 82:4394-4398) was linearized with Sma 1, phenol/$CHCl_3$ extracted, ethanol precipitated, 70% ethanol washed, dried and redissolved in 10 mM Tris, 1 mM EDTA, pH 8.0. Sma 1 cuts at a site 280 bp downstream from the transcription inhibition site. 500 ng (approximately 30 nM final concentration) of the linearized G-less cassette was hybridized with 1-10 μM oligomers capable of forming triple helix DNA in the motifs described. Hybridization was performed in 50 mM KCl, 5 mM $MgCl_2$, 1 mM spermine, and 20 mM Hepes, pH 7.6 in a total volume of 5 μL at room temperature for one hour. 13 μg of Jurkat nuclear extract was then added, and the transcription initiation complex was allowed to

form at room temperature for thirty minutes. 630 μM of ATP and CTP, 50 μM 3'-o-methyl GTP, 1 μM UTP, and 10 μCi α ^{32}P UTP was added to initiate transcription for a final reaction volume of 10 μL and incubated at 30°C for thirty minutes. The reactions were terminated with 140 μL of 50 mM Tris, pH 7.5, 1% SDS, 5 mM EDTA; extracted with phenol/$CHCl_3$; precipitated with 10 μg t-RNA, 0.2 M NH_4OAc, pH 5.2, and 2 volumes of ethanol; washed with 70% ethanol; dried; and redissolved in 90% formamide, 90 mM Tris, 90 mM Borate, 2.5 mM EDTA, pH 8.3. The samples were analyzed by autoradiography after electrophoresis through a 8.3 M urea/6% polyacrylamide gel.

Example 1

Synthesis of ODNs used to form Triplexes

ODNs 1, 2 and 6 (Table 1) were synthesized using conventional methods of solid phase synthesis as described above and well known in the art. Methods for such synthesis are found, for example, in Froehler, B., et al., Nucleic Acids Research (1986) 14:5399-5467; Nucleic Acids Research (1988) 16:4831-4839; Nucleosides and Nucleotides (1987) 6:287-291; Froehler, B., Tet Lett (1986) 27:5575-5578.

1.a. Synthesis of ODN 3

ODN 3, as shown in Table 1, contains the base analog N6-methyl-8-hydroxy-2'-deoxyadenosine (designated Z). This analog is able to function in both the CT and GT motifs. This analog was synthesized as described below. Following synthesis, the base analog was incorporated into the growing oligonucleotide chain using conventional methods of solid phase synthesis. After cleavage from the support, the ODN was extracted in butanol.

Preparation of N6-methyl-8-bromo-2'-deoxyadenosine

A solution of 2.45g of N6-methyl-2'-deoxyadenosine (Jones, J.W., et al., J. Am. Chem. Soc. (1963) 85:193-201) in 50 mL of water was treated with 50 mL of 0.5M sodium acetate/acetic acid buffer (pH 4.65) and 75 mL of saturated bromine water. After 5 hrs of stirring, sodium bisulfite was added to the thick suspension until it became colorless. The pH was then adjusted to 7 with 10M sodium hydroxide and the mixture was heated until a clear solution was obtained. The solution was allowed to cool, and the resulting precipitate was collected by filtration to afford 1.4g of the title compound.

Preparation of N6-methyl-N6-acetyl-8-hydroxy-2'-deoxyadenosine

A suspension of 2.1g of N6-methyl-8-bromo-2'-deoxyadenosine and 2.1g of sodium acetate in 20 mL of acetic anhydride was heated at 120°C for 14 hr. The mixture was poured onto ice and extracted with ethyl acetate. The organic extracts were washed with water, saturated sodium bicarbonate solution and brine, dried over magnesium sulfate, filtered and evaporated. The residue was dissolved in 10 mL of methanol and the solution was treated with 5 mL of 1M sodium methoxide solution. After 10 min, the solution was neutralized with Dowex 50X H^+ resin, filtered and evaporated. The residue was chromatographed on a silica gel column using acetonitrile/water 9:1 v:v to afford 660 mg of the title compound as a crisp yellow foam.

Preparation of N6-methyl-8-hydroxy-2'-deoxyadenosine

A solution of 130 mg of N6-methyl-N6-acetyl-8-hydroxy-2'-deoxyadenosine in 3 ml of 1N methanolic sodium

methoxide was stirred for 72 h. The mixture was neutralized with Dowex 50XH$^+$ resin, the resin was removed by filtration and was washed with methanol. The combined filtrates were evaporated, and the residue was crystallized from ethanol to yield 28 mg of the title compound.

1.b. Synthesis of ODN 4

ODN 4, as shown in Table 1, contains 2 base analogs: N6-methyl-8-hydroxy-2'-deoxyadenosine and (2) 5-methylcytosine (designated W). N6-methyl-8-dydroxy-2'-deoxyadenosine was synthesized as described in Example 1.a. 5-methylcytidine is available commercially from Sigma. This is converted to the 5'-DMT 3'-hydrogen phosphonate monomer synthon according to standard methods. The base analogs were incorporated into the growing chain where indicated in Table 1 using standard techniques.

1.c. Synthesis of ODN 5

ODN 5, as shown in Table 1, includes the base analogs aziridinylcytosine (N4,N4-ethanocytosine) and anthraquinone/pseudonucleotide. These analogs were synthesized as described below. Following synthesis, the base analogs were incorporated into the growing oligonucleotide chain using conventional methods of solid phase synthesis. C groups were FMOC protected during synthesis of the oligomer and an oxalyl-CPG support (R. Letsinger, personal communication, described below) was used during synthesis.

I. Preparation of aziridinylcytosine

The synthesis scheme for aziridinylcytosine is shown in Figure 1. The steps in the synthesis refer to Webb and Matteucci, Nucleic Acids Res (1986) 14:5399-

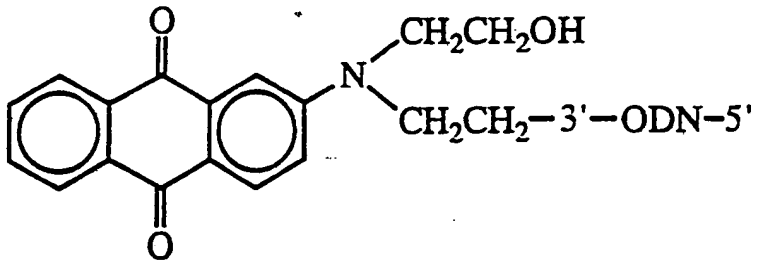
5467, Froehler and Matteucci, Nucleic Acids Res (1986) 14:7661-7674 and Marugg et al., Tet. Lett. 27:2661.

After synthesis, the base analog was coupled to a CPG support for the production of ODN 5 using the following method (R. Letsinger, personal communication). Oxalyl chloride (20 μl, 0.23 mmol) was added to a solution of 1,2,4-triazole (77 mg, 1.1 mmol) in acetonitrile (2 ml). A small amount of precipitate formed but disappeared after addition of pyridine (0.1 ml). The DMT-nucleoside (0.23 mmol) in acetonitrile (1 ml) and pyridine (0.5 ml) was added, and after one hour the solution was drawn into a syringe containing aminopropylsilyl-controlled-poreglass (CPG) (400mg; 80-100 mesh, 500 A pore). This mixture was allowed to stand for 15 min. and the liquid was ejected and the solid washed four times with acetonitrile. Any residual amino groups were capped by drawing in equal volumes of THF solutions of DMAP (0.3 M) and acetic anhydride (0.6 M). The support was then washed with pyridine and acetonitrile and dried.

After ODN 5 was synthesized, the support bound H-phosphonate oligomer was oxidized with I_2/pyridine/H_2O twice for 30 min and subsequently converted to the aziridine compound by deprotection and cleavage from the support by treatment with 20% aziridine in DMF for 2 hours at room temperature. The oligomer was recovered and further purified by running the reaction mixture from the synthesis machine over NAP-5 (Pharmacia Sephadex G-25) column to remove salts, free aziridinylcytosine residues, FMOC blockers, etc. The NAP-5 column was used according to the manufacturers directions.

II. Preparation of Anthraquinone/pseudonucleotide

Anthraquinone/pseudonucleotide has the following structure.



Anthraquinone/pseudonucleotide was synthesized as follows:

Preparation of Protected Pseudonucleoside Conjugated to Anthraquinone

A. 2-(N,N-diethanolamino)anthraquinone was prepared as follows. A mixture of 2-chloroanthraquinone (2.42 g; 10 nmole) and an excess of diethanolamine in DMSO (20 ml) was heated to 150°C. After 24 hours reaction, the reaction mixture was cooled to room temperature, then poured into water (70 ml). The red precipitate was filtered off, washed thoroughly with water, and dried in air. The crude product, containing some starting material, was used for the protection reaction of paragraph B without further purification.

B. The crude compound prepared in paragraph A, was dissolved in pyridine (20 ml) and triethyl amine (1.7 ml), cooled to 0°C, followed by addition of DMAP (0.2 g) and DMT-Cl (4.0 g; 12 mmol). The reaction mixture was warmed to room temperature. After 4 hours of reaction, more DMT-Cl (1 g) was added to the reaction mixture and reacted one more hour, then concentrated to dryness. The residue was then partitioned between methylene chloride and saturated sodium bicarbonate solution. The organic solution was separated and dried, purified by flash column chromatography on silica gel, eluted with 1% Et_3N/1% CH_3OH/CH_2Cl_2, to afford the product mono-DMT-protected 2-N,N-diethanolaminoanthraquinone, in an amount of 0.7 g (13% overall yield); 3.1 g of unreacted starting material was recovered.

Conjugation to CPG Support

A. A mixture of the DMT-protected anthraquinone pseudonucleoside of Example 1, paragraph B (0.5 g; 0.81 mmol), DMAP (0.1 g) and succinic anhydride (0.326 g; 3.26 mmol) in pyridine (10 ml) was stirred at room temperature for 4 hours, and more succinic anhydride (0.1 g) was added to the reaction. After 2 more hours of reaction, the residue was dissolved in methylene chloride, washed with 1M TEAB aqueous solution. The organic solution was isolated, dried over Na_2SO_4, concentrated, then purified by flash column chromatography, and eluted with 1% Et_3N/2% CH_3OH/CH_2Cl_2, 1% Et_3N/5% CH_3OH/CH_2CH_2, to afford the succinylated product. The succinylated form of the subject compound (0.39 g) was isolated as a red solid (yield 59%).

B. After succinylation as described in paragraph A, 3-5 equivalents of the succinylated pseudonucleoside, 10 equivalents of diisopropyl-carbodiimide, a catalytic amount of DMAP and CPG in DMF/pyridine (4/1; 4 ml/g CPG) were shaken at room temperature overnight and then capped with acetic anhydride and pyridine. After 4 hours capping at room temperature, quenching by slow addition of methanol, CPG was filtered off and washed thoroughly with methylene chloride, methanol, and ether, and dried under vacuum overnight. The resulting CPG derivative with anthraquinone-coupled pseudonucleoside were then used to provide the pseudonucleoside at the 3' terminus of an oligonucleotide.

1.d. Synthesis of ODN 7

ODN 7, as shown in Table 1, includes the base analog 5-methylcytidine. This analog is commercially available from Sigma and was converted to the 5'-DMT 3'-hydrogenphosphonate N4-benzpyl synthon and then incorporated into the growing chain by standard oligonucleotide chemistry.

Example 2

Transcription Truncation by GT Motif ODN

ODN 1 and 2 were separately hybridized to the ODN 1 target sequence at 24°C for 30 min in 140 mM KCl, 5 mM $MgCl_2$, 50 mM HEPES, pH 7.2, 1 mM spermine to allow formation of triplex structures. Transcription complexes were formed at 30°C for 30 minutes followed by addition of nucleotides to initiate transcription and ribonuclease T1 (to eliminate background transcripts, the nuclease cuts RNA at G residues, hence G-less system), which was run for 30 minutes. Full-length transcription yields a 280 base RNA; the template was linearized by a restriction enzyme cut 280 bases downstream from the initiation site. The ODN 1 target sequence was located at a region 190 bases downstream from the initiation site. ODN 2 is a mismatched control having a similar base composition but does not form a triplex with the target DNA sequence. Autoradiograms of labeled transcripts show both full-length (280 base) and truncated (190 base) transcript using 10 μM ODN 1. Only full-length (280 base) transcript was seen with ODN 2 used at 10 μM. The truncated transcript consisted of several discrete bands that ranged from about 187 to 193 bases in length, suggesting that RNA polymerase was blocked at several sites near the promoter-proximal end of the triplex target site. The ratio of truncated to full-length transcript was greater than approximately 0.2

at all concentrations of ODN 1. Concentrations of ODN 1 or ODN 2 above 10 μM led to nonspecific inhibition of transcription.

Example 3

Transcription Truncation by GT Motif ODN, Formation of Triplex in Cellular Extracts

ODNs 1 and 6 were used at 5 μM to hybridize as above, except that the buffer contained 50 mM KCl instead of 140 mM. Triplex was formed at three different times: (i) 30 min before addition of cell extract; (ii) simultaneously with addition of cell extract; and (iii) 2 minutes after initiation of transcription by addition of nucleotides. Addition of cell extract permits formation of transcription initiation complexes. Blocking of transcription by ODN 1 was expected to generate a 190 base transcript, while a full-length transcript of 280 bases results from unimpeded transcription. In all cases, a truncated transcript of 190 bases was observed. The ratio of 190:280 base transcript for (i), (ii) and (iii) was approximately 0.5 in each case. Truncated transcripts were observed as discrete bands as described in Example 2. Addition of 5 μM control ODN 6 at 30 min prior to addition of cell extract and simultaneously with addition of cell extract, did not lead to any detectable truncation of transcription. Addition of ODN at (ii) and (iii) and truncation of transcription demonstrates that progression of transcription may be blocked both during and before RNA polymerization under conditions that are similar to those found in mammalian cells. Thus, proteins and other cell components present in the nuclear extract did not inhibit the formation of triple helical DNA.

Example 4

Transcription Truncation by CT Motif ODN

ODN 3 and control ODN 7 were used to form triplex structures with a target sequence that was 190 bases downstream from the transcription initiation site. ODN 3 contains N6-methyl-8-hydroxy-2'-deoxyadenosine residues in place of cytosine residues that bind to guanosine residues in target duplex DNA at physiological pH. Hybridization at pH 7.2 generated a triplex structure in the target DNA and led to generation of truncated transcripts approximately 190 bases long. Truncated transcripts were observed as discrete bands as described in Example 2. Only full-length transcripts were observed in the reaction mixture that was prehybridized with ODN 7.

ODN 4, which contains 5-methylcytosine in place of cytosine residues, was hybridized for 0.5, 1.0 and 2.0 hours at 25°C and 20 μM to duplex target DNA at pH 6.0 to generate triplex structures. The pH was adjusted to 7.2 and cell extract was added followed by addition of nucleotides to initiate transcription as described in Examples 2 and 3. ODN 4, which does not form a triplex with duplex target DNA at pH 7.2, was used as a control by prehybridizing at pH 7.2 prior to addition of cell extract and nucleotides. The target sequence for ODN 4 was 68 bases downstream from the transcription initiation site. Truncated transcripts were observed from reactions that were prehybridized at pH 6.0 but not from mixtures prehybridized at pH 7.2. Truncated transcripts approximately 68 bases long were observed as discrete bands as described in Example 2. This result shows that the preformed triplex was capable of blocking transcription utilizing CT motif DNA binding. Generation of truncated transcripts at pH 7.2 demonstrates that the triplex structure formed by ODN 4 at pH 6.0 was stable

enough to block transcription under pH conditions where the ODN would not form a triplex structure.

Example 5

Transcription Truncation by CT Motif ODN Containing A Covalent DNA Alkylating Base Analog

ODN 5, containing the cytosine base analog, aziridinylcytosine, was used to form triplex complexes with a target DNA sequence located 60 bases downstream from the transcription start site. Triplex hybridization and alkylation of target duplex was carried out at pH 6.0 for 120 min at 25°C using 10 μM ODN 5 and the pH was then adjusted to pH 7.2 for addition of cell extract and then 30 min later nucleotides were added. The transcription reaction was carried out for 30 min at 30°C. A truncated transcript approximately 60 bases long was observed at a truncated to full-length ratio of about 0.3. ODN 5 was used to cleave the template to demonstrate crosslinking to the target sequence. The pyrrolidine reaction demonstrated that about 20% of ODN 5 had formed a covalent link with the target DNA. ODN 1 was used as a control for nonspecific transcription inhibition. In this case, no target sequence was present in the transcribed DNA. No truncated transcripts were observed from mixtures incubated with ODN 1.

Thus, methods for inhibiting transcription by forming triple helices with transcribed regions of DNA are described. Although preferred embodiments of the subject invention have been described in some detail, it is understood that obvious variations can be made without departing from the spirit and the scope of the invention as defined by the appended claims.

Claims

1. A method to inhibit transcription of duplex DNA, which comprises contacting duplex DNA having a control region and a transcribed region with an amount of oligomer effective to bind to said transcribed region, thereby forming a triple helix.

2. The method of claim 1 wherein said transcribed region is within an exon.

3. The method of claim 1 wherein said transcribed region is within an intron.

4. The method of claim 1 wherein said transcribed region contains both exon and intron sequences.

5. The method of claim 1 wherein said oligomer forms a triple helix with said duplex DNA using the CT motif.

6. The method of claim 1 wherein said oligomer forms a triple helix with said duplex DNA using the GT motif.

7. The method of claim 1 wherein said oligomer comprises regions of inverted polarity.

8. The method of claim 1 wherein said oligomer is at least 10 nucleotides in length.

9. The method of claim 1 wherein said oligomer is about 10 to about 25 nucleotides in length.

10. The method of claim 1 wherein said oligomer includes at least one base analog.

11. The method of claim 10 wherein said at least one base analog is an alkylating base analog.

12. The method of claim 11 wherein said at least one base analog is an aziridinyl nucleotide.

13. The method of claim 12 wherein said aziridinyl nucleotide is aziridinylcytosine.

14. The method of claim 10 wherein said at least one base analog is an anthraquinone derivatized oligonucleotide.

15. The method of claim 10 wherein said at least one base analog is N6-methyl-8-hydroxy-2'-deoxyadenosine.

16. The method of claim 10 wherein said at least one base analog is 5-methylcytosine.

17. A method of inhibiting RNA polymerase by a major groove binding agent, said method comprising binding said major groove binding agent to transcribed regions of DNA in a sequence-specific manner.

18. The method of claim 17 wherein the RNA polymerase in RNA polymerase II.

19. A DNA triplex comprising:

a) duplex DNA having a control region and a transcribed region; and

b) a single-stranded DNA oligomer coupled to a portion of said transcribed region.

20. The triplex of claim 19 wherein said transcribed region is within an exon.

21. The triplex of claim 19 wherein said transcribed region is within an intron.

22. The triplex of claim 19 wherein said transcribed region contains both exon and intron sequences.

23. The triplex of claim 19 wherein said oligomer is at least 10 nucleotides in length.

24. The triplex of claim 19 wherein said oligomer is about 10 to about 25 nucleotides in length.

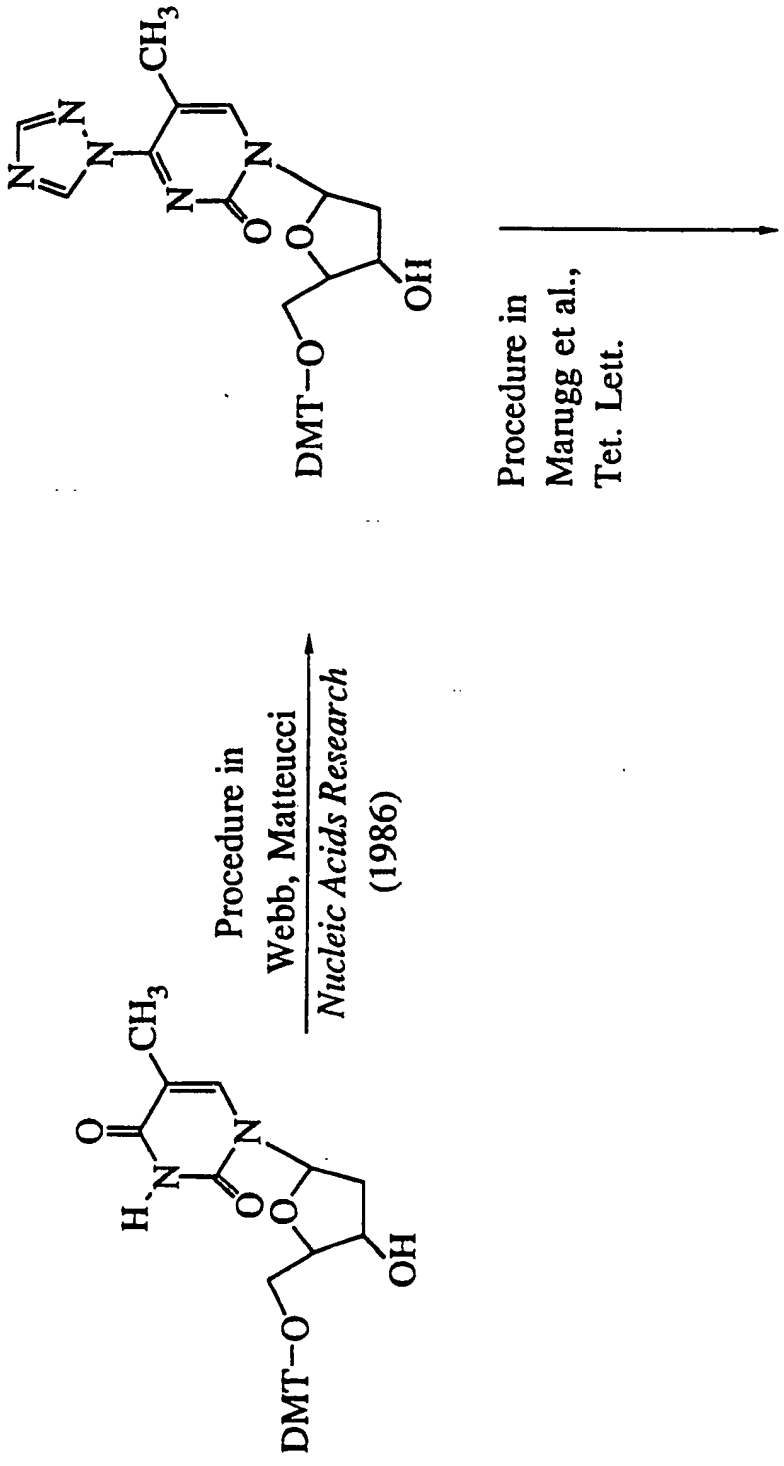

Procedure in
Webb, Matteucci
Nucleic Acids Research
(1986)
Procedure in
Marugg et al.,
Tet. Lett.
DMT–O
OH
DMT–O
OH

Figure 1

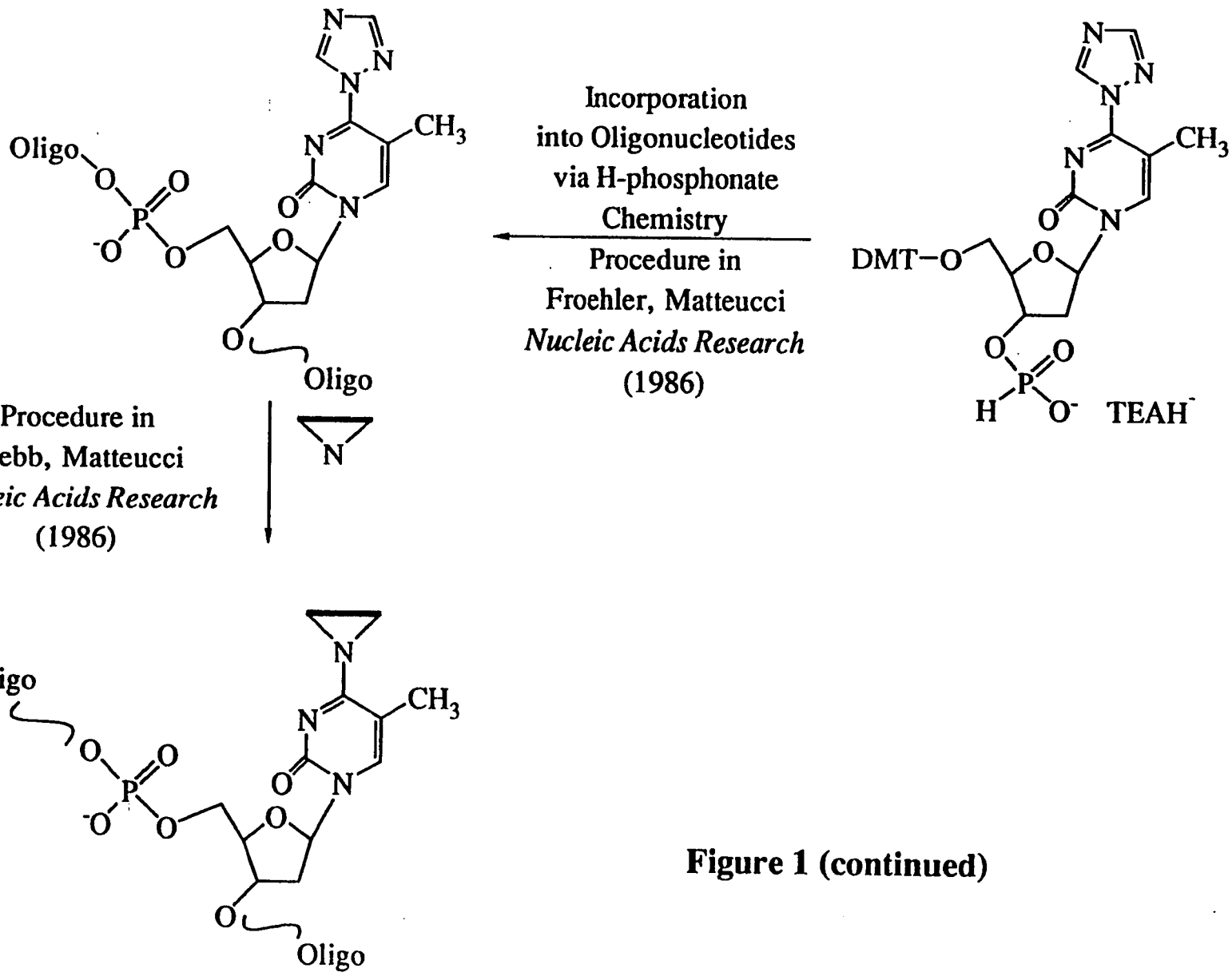


Figure 1 (continued)

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicat all)[3]

According to International Patent Classification (IPC) or t both National Classification and IPC

IPC (5): C12Q 1/68; C12P 21/00; C12N 15/00
US CL : 435/6; 536/27; 935/33; 435/68; 435/172.1

II. FIELDS SEARCHED

Minimum D cumentation Searched[4]

Classification System	Classification Symb ls
U.S.	435/6; 536/27; 935/33; 435/68; 435/172.1

Documentation Searched other than Minimum Documentation
to the extent that such Documents are included in the Fields Searched[5]

APS

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
A	Journal of Experimental Pathology; Volume 2, number 3, issued 1985, K. Minton "The Triple Helix: A Potential Mechanism for Gene Regulation" pages 135-147, see abstract.	1-24
X/Y	Proc. Natl. Acad. Sci., volume 85, issued March 1988, Proseuth et al., "Sequence-specific binding and photocrosslinking of alpha and beta oligodeoxnucleotides to the major groove of DNA via triple-helix formation," pages 1349-1353, see abstract, final paragraph of column 1 page 1353.	19-24/1-18
Y	J. Cell Biochem Supp.; vol. 12, Part D, issued 1988, Kohuri et al. "Homopurine-Homopyrimidine sequences formation of the triple helical structure is supercoiled plasmid DNA and effects on the level of gene epxression" see abstract no. 88-430278.	19-24

* Special categories of cited documents:[15]

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completi n of the Internati nal Search[2]	Date of Mailing of this International Search Report[2]
10 MARCH 1992	02 APR 1992
International Searching Authority[1]	**Signature of Authorized Offi er[20]**
ISA/US	Scott A. Chambers

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X/Y	Proc. Natl. Acad. Sci., volume 85, issued September 1988; Hanvey et al. "Intramolecular DAN triplexes in supercoiled plasmids" pages 6292-6296, pag 6296 column 1.	1,5,6,8,9,17-19,23,24/2-4,7,10-16, 20-22
Y	Scienc , volume 245, issued 18 August 1989, Maher et al., "Inhibition of DNA binding Proteins by Oligonucleotide-Directed Triple Helix Formation" pages 725-730, see abstract.	1-24

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers _, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers _, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.